The combined use of chemical and biochemical markers in *Rutilus rutilus* to assess the effect of dredging in the lower course of the Ebro River

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Abstract

The lower course of the Ebro River is polluted with high concentrations of organochlorine compounds dumped by a chloro-alkali plant during the last century. A remediation plan, including building of a protective wall, removal and disposal of polluted sediments started in 2012. With the aim of assessing the effects of dredging of contaminated sediments and potential alterations of water quality, areas located upstream (RR) and downstream (BE, A) the chemical plant (FL) were monitored prior (October 2012) and during dredging (June 2013) using roach (*Rutilus rutilus*) as sentinel organisms. Concentrations of organochlorine compounds (OCs) in fish muscle and biliary levels of polycyclic aromatic hydrocarbons (PAHs), galaxolide (HHCB) and alkyphenols (APEs) were determined together with selected enzymatic activities (7-ethoxyresorufin-*O*-deethylase (EROD), 7-benzyloxy-4-trifluoromethyl-coumarin *O*-debenzyloxylase (BFCOD) and UDP-glucuronyltransferase (UGT)) in the liver. The obtained results proved the effectiveness of the wall retaining suspended particles and avoiding further contamination of downstream sites as fish sampled at downstream sites showed up to 9-fold higher concentrations of OCs in muscle during wall construction than during dredging. EROD and UGT activities were induced in fish from downstream sites; however, no clear response to the observed pollution gradient was detected.

Key words: organochlorine compounds; biomarkers; bile; EROD, BFCOD.

1. Introduction

The Ebro River (NE Spain) with a drainage area of about 85,000 km² and a population of three million people living in the watershed, receives significant amounts of industrial and urban discharges together with agricultural inputs (Fernández et al., 1999; Claver et al., 2006; Silva et al., 2011). Flix, located in the low course of the river, is one of the most heavily polluted areas due to discharges from a chloro-alkali plant. This area has received over a hundred years industrial wastes containing organochlorine compounds (OCs) (hexachlorobenzene (HCB), pentachlorobenzene (PeCB), dichloro-diphenyltrichloroethane (DDTs), polychlorobiphenyls (PCBs), polychloronaphtalenes (PCNs) and polychlorostirens (PCEs)), and metals (Cd, Hg), among other pollutants (Suárez-Serrano et al., 2010). This resulted in the accumulation of 500,000 tons of industrial wastes in the adjacent riverbed and the consequent contamination of river sediments with concentrations of PeCB, PCBs, DDTs, PCNs and Hg in the range of 1 to 640 µg/g (Grimalt et al., 2003; Palanques et al., 2014). Moreover, high concentrations of PCBs, PeCBs, HCB, HCHs, DDE and DDTs have also been reported in sediments from downstream areas, confirming the transport of pollutants to the lower part of the River (Bosch et al., 2009). In addition, significant amounts of OCs and Hg have been reported in biota from Flix and downstream (Lavado et al., 2006; Faria et al., 2010; Soto et al., 2011; Alcaraz et al., 2011; Huertas et al., 2016).

Biomarkers have been successfully applied in Mediterranean rivers to monitor the environmental status and the potential effects of pollutants in aquatic organisms (Colin et al., 2016). Thus, a significant increase in EROD activity together with a depletion of acetylcholinesterase (AChE) activity and high levels of metallothioneins have been reported in carps from Flix and associated to exposure to PCBs, DDTs and

nonylphenol and Hg, among other pollutants (Lavado et al., 2006). Elevated CYP1A gene expression was detected in barbels and carps from Flix and related to exposure to dioxin-like PCBs and other OCs (Olivares et al., 2010, Eljarrat et al., 2008). Similarly, Faria et al. (2010) reported high EROD activity, lipid peroxidation and DNA damage in mussels and crayfish from the area.

Due to this severe pollution, an ambitious remediation plan started in 2010 with the construction of a retaining wall to isolate the contaminated sludge. The building of the retaining wall finished in 2012 and dredging of the contaminated sediments started in March 2013. These sediments were removed, subsequently processed in a nearby treatment plant and disposed in a landfill.

As dredging activities could represent a considerable release of toxic compounds accumulated in sediments to the river water, posing a risk to aquatic organisms, this study was designed to investigate the potential impact of dredging in aquatic fauna using roach (*Rutilus rutilus*) as sentinel species, as this species has been successfully used in a number of biomonitoring studies all over Europe to assess the impact of water quality in aquatic fauna (Bjerregaard et al., 2006; Gerbron et al., 2014). Sampling campaigns were performed in October 2012 (wall construction) and in June 2013 (during dredging) in four representative stations: Ribarroja, a potential reference site located upstream of Flix (Navarro et al., 2009); Flix, the main focus of pollution, and two downstream stations, Benifallet and Amposta. Combined chemical analysis of selected contaminants in muscle and bile, together with histological examination of the gonads and several biochemical markers including 7-ethoxyresorufin *O*-deethylase (EROD) activity, CYP3A catalytic probe (BFCOD) and phase II enzyme UDP-glucoronyltransferase (UGT)) were selected for the study, since they are induced by a

variety of xenobiotics, and catalyze the oxidative metabolism and conjugation of both xenobiotics and endogenous compounds.

2. Material and methods

2.1. Chemicals

Uridine 5'-diphosphoglucuronic acid (UDPGA), *p*-nitrophenol (pNP), NADPH, 7-ethoxyresorufin (7-ER), bovine serum albumin (BSA; fatty acid free, ≥ 99% purity), methyl tert-butyl ether (MTBE) and hydroxylamine hydrochloride were obtained from Sigma-Aldrich (Steinheim, Germany). 7-Benzyloxy-4-trifluoromethyl-coumarin (7-BFC) was purchased from Cypex (Dundee, Scotland, UK). Cellulose extraction cartridges were obtained from Whatman Ltd. (UK). External standard mixtures of PeCB, HCB, HCHs (α-HCH, β-HCH, γ-HCH and δ-HCH), DDTs (2,4'-DDE, 4,4´-DDE, 2,4'-DDD, 4,4'-DDD, 2,4'-DDT, 4,4'-DDT) and PCBs (congeners 28, 52, 101, 118, 138, 153 and 180) and the internal standards PCB 200 and PCB 142 were purchased from Dr. Ehrenstofer (Wesel, Germany). 1,2,4,5-tetrabromobenzene (TBB) was from Aldrich-Chemie (Steinheim, Germany) and all solvents and other reagents were obtained from Merck (Darmstadt, Germany).

2.2. Sample collection and preparation

Male and female roach (*Rutilus rutilus*) were collected by DC electrofishing from four stations along the Ebro River: Ribarroja (RR) a relatively clean site located 6 km upstream from the dredging site; Flix (FL), the historically polluted site; Benifallet (BE)

and Amposta (A), located 42 and 82 km downstream from Flix, respectively (Fig. S1). Samplings were carried out in October 2012 and June 2013. Immediately after collection, fish were killed by severing the spinal cord. Total length and weight were measured (Table S1, supplementary information). Liver, muscle and bile were dissected and immediately frozen in liquid nitrogen, and stored at -80 ºC. A subsample from the central part of gonad tissue was fixed in 10% formaldehyde buffered with 100 mM sodium phosphate at pH 7.4 for histological examination.

2.3. Histological analysis of gonads

Gonad samples fixed in 10% formalin for 24 h were dehydrated with ethanol, cleared in Histo-Clear (National Diagnostic, Atlanta, USA) and embedded in paraplast (Sigma–Aldrich, Steinheim, Germany). Tissue sections (7 µM) were stained with haematoxylin-eosin Y and examined by light microscopy. Gonads were sorted into four stages of sexual maturation (0- undeveloped, I- early maturation, II -mid maturation, III -final maturation) following a modification of Geraudie et al. (2010).

2.4. Chemical analysis

Analysis of bile samples

Hydroxylated metabolites of PAHs, alkylphenols and galaxolide (HHCB) were determined in bile samples following the method described in Escartín and Porte (1999) with some modifications. Briefly, 20 to 100 mg of bile were incubated for 1 h at 40ºC in 0.4 M acetic acid/sodium acetate buffer pH 5.0 containing 2000 units of β-

147 glucoronidase and 50 units of sulphatase. Hydrolysed metabolites were extracted with
148 ethyl acetate; the extracts were recombined and concentrated under a nitrogen stream.
149 Dry residues were derivatized by the addition of 100 μL of bis-
150 (trimethylsilyl)trifluoroacetamide (BSTFA), heated for 1 h at 70ºC, dried and
151 reconstituted in ethyl acetate (1:10, w:v). Analyses were carried out by gas
152 chromatography-mass spectrometry (GC-MS) operating in electron impact (EI) and
153 selected ion monitoring (SIM) modes. The equipment was an Agilent 6890 series GC
154 system with an Agilent 5973 Network mass selective detector. The column, a TRB-
155 5MS 30 m x 0.20 mm i.d., film thickness 0.25 μm (Teknokroma Analítica SA Spain),
156 was programmed from 90ºC to 140ºC at 10°C/min and from 140ºC to 300ºC at 4
157 ºC/min. The carrier gas was Helium at 80 Kpa. The injector temperature was 250°C and
158 the ion source and the analyser were maintained at 230°C and 150°C, respectively.
159 Target compounds were identified by comparison of the retention times and spectra of
160 reference compounds, namely 1-naphthol, 1-pyrenol, 4-nonylphenol (NP), 4-*tert*-
161 octylphenol (OP) and HHCB (Sigma-Aldrich, Steinheim, Germany). The ions of silyl-
162 derivatives used for monitoring and quantification were: *m/z* 216, 201 for 1-naphthol,
163 *m/z* 290 for 1-pyrenol, *m/z* 207,193 for NP, *m/z* 207 for OP and *m/z* 243, 258 for HHCB.
164 Quantification was performed by external standard method. Concentrations for all
165 compounds are expressed as ng/mL of bile.
166
167 **Analysis of organochlorine compounds in muscle**
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169 Three pooled samples (between 2 and 3 individuals per pool) of fish muscle were
170 analyzed for each sampling site. The extraction of organic pollutants was performed as
171 described in Koenig et al., (2013). Briefly, lyophilized muscle tissue (2-4 g) was ground

and homogenized with anhydrous Na_2SO_4 and soxhlet-extracted with 100 mL of dichloromethane: hexane (1:4) for 18 h. TBB (1,2,4,5-tetrabromobenzene) and PCB 200 were added as recovery standards. Extracts were further purified with concentrated sulfuric acid. The cleaned extracts were concentrated by evaporation, transferred to vials, evaporated to near dryness under a gentle stream of nitrogen and re-dissolved in 100 µL of PCB 142 in isooctane, as internal standard, prior to the determination of OC levels.

To determine levels of PCBs (7 congeners: IUPAC # 18, 52, 101, 118, 138, 153, 180), DDTs (2,4'-DDE, 4,4´-DDE, 2,4'-DDD, 4,4'-DDD, 2,4'-DDT, 4,4'-DDT), PeCB, HCB and HCHs (α-, β-, γ-, δ-HCH), samples were analyzed using a gas chromatograph (Model HP-6890) equipped with an electron-capture detector (µ-ECD). A 60 m x 0.25 mm DB-5 column (J&W Scientific, Folsom, CA, USA) coated with 5% diphenylpolydimethylsiloxane (film thickness 0.25 µm) was used for separation. The oven temperature was programmed to increase from 90°C (holding time 2 min) to 130°C at a rate of 15°C/min and finally to 290°C at 4°C/min, holding the final temperature for 20 min. The injector and detector temperatures were 280°C and 320°C, respectively. Injection was performed in splitless mode and helium was used as carrier gas (30 psi). OC levels were determined by internal standard method. Procedural blanks were performed for every set of six samples. Blank values were used to establish method detection (MDL) and quantification limits (MQL), which were defined as the mean of the blanks plus three times (MDL) or five times (MQL) the standard deviation. They were in the order of 0.02 and 0.59 ng/g d.w. (MDL) and 0.02 to 0.91 ng/g d.w. (MQL), depending on the compound. Extraction and analytical performances were evaluated by surrogate standard recoveries, which were 58±10% and 80±23% for TBB

and PCB 200, respectively. Values reported in this study were corrected by surrogate recoveries.

2.5. Enzymatic activities

After weighing, livers were flushed with ice-cold 1.15% KCl and homogenized in 1:4 w/v of 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, 150 mM KCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500 x *g* for 15 min, and the obtained supernatant centrifuged at 12,000 x *g* for 20 min at 6ºC. The resulting supernatant was further centrifuged at 100,000 x *g* for 60 min at 6ºC to obtain the microsomal pellet, which was resuspended in a ratio of 0.5 mL buffer/g of liver in 100 mM potassium–phosphate buffer pH 7.4, containing 150 mM KCl, 20% (w/v) glycerol, 1 mM DTT, 0.1 mM PMSF and 1 mM EDTA. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard.

7-Ethoxyresorufin *O*-deethylase (EROD) activity was assayed by incubating 0.1 mg of liver microsomal protein with 3.7 μM of 7-ethoxyresorufin and 225 μM of NADPH in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 at 30ºC for 10 min. The reaction was stopped by adding 400 μL of ice-cold acetonitrile. After centrifugation, an aliquot of the supernatant was transferred into a 96-multiwell plate. Fluorescence of 7-hydroxyresorufin was read at the excitation/emission wavelength pairs of 537/583, using a Varioskan microplate reader (Thermo Electron Corporation). Quantification was performed using a 7-hydroxyresorufin calibration curve and the activity calculated as the amount of 7-hydroxyresorufin (pmol) generated per milligram of protein per minute.

Benzyloxy-4-trifluoromethyl-coumarin-O-debenzyloxylase (BFCOD) activity was analysed according to the procedure described by Thibaut et al. (2006). The assay consisted in incubating 25 μg of liver microsomal protein with 200 μM of 7-benzyloxy-4-trifluoromethyl-coumarin (BFC) and 22.5 μM of NADPH in 100 mM potassium phosphate buffer pH 7.4 at 30ºC for 10 min. The reaction was stopped by addition of acetonitrile (20:80, v/v) and the fluorescence was read in a 200 μL aliquot transferred into a 96-multiwell plate at the excitation/emission wavelength pairs of 409/530 nm, using a Varioskan microplate reader. The activity was calculated as the amount of 7-hydroxy-4-(trifluoromethyl)-coumarin (pmol) generated per milligram of protein per minute.

Hepatic UDP-glucuronyltransferase (UGT) was assayed by a modification of the method described in Clarke et al. (1992). Briefly, 0.25 mg of liver microsomal proteins pre-treated with Triton X-100 were incubated with 3.15 mM of UDPGA in 30 mM Tris/$MgCl_2$ buffer pH 7.4. The reaction was initiated by the addition of 80 μM p-nitrophenol (pNP), run for 30 min at 30ºC and stopped by the addition of 0.2 M ice-cold trichloracetic acid, centrifuged (1,500 x *g*; 15 min), alkalinized with 0.1 mL of 10 N KOH and the remaining pNP measured spectrophotometrically at 405 nm. The activity was calculated as the amount of pNP (nmol) consumed per milligram of protein per minute of reaction time.

2.6. Statistical analysis

Enzymatic activities were determined individually in 6-12 organisms per site and run per duplicate. Chemical analyses were conducted in pooled muscle tissue of three individuals (n = 2 to 3 pools per site) and bile samples were analyzed individually (n = 5

to 9). Values are presented as mean ± SEM. Prior to assessing temporal and spatial differences, data was tested for normality and homogeneous variance (Levene's test). Enzymatic activities and organochlorine content followed a normal distribution and two-way ANOVA followed by multiple independent group comparison (Tukey's test) was used for statistical analysis. For bile metabolites, the assumption of normality and homogeneous variance were not meet and non-parametric analyses (Kruskal Wallis followed by Mann-Whitney U test) were used for statistical analysis. All statistical analyses were performed with the software package SPSS 15.0 (SPSS Inc., Chicago, IL) and STATA SE/12.1. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Morphometric data of samples

Length, weight and condition factor (CF) of the sampled fish are reported in Table S1. Fish collected in RR in October 2012 were significantly bigger (13.8 ± 0.3 cm; 29.5 ± 0.7 g) than those from A (11.5 ± 0.6 cm; 22.5 ± 4.0 g); while in the second sampling (June 2013), fish from A were significantly bigger (16.3 ± 0.3 cm and 67.5 ± 3.5 g) than the rest. Nonetheless, no significant differences were observed among sampling sites in terms of CF of the sampled individuals, indicating a similar nutritional state of the fish.

3.2. Analysis of bile samples

The concentration of organic contaminants measured in bile is shown in Figure 1. NP was the most abundant pollutant detected (450-2250 ng/mL bile), followed by the

synthetic fragrance HHCB (57-280 ng/mL bile). No significant differences among sampling sites were observed for the levels of NP, OP, 1-naphtol and 1-pyrenol in fish bile in any of the two samplings. In contrast, high levels of HHCB were measured in October 2012 in fish from FL (195 ± 67 ng/mL bile) in comparison to BE (57 ± 7 ng/mL bile), while in June 2013, the highest concentration of HHCB was detected in the bile of fish from RR (281 ± 44 ng/mL bile) and the lowest at A (157 ± 15 ng/mL bile).

When comparing the levels of chemicals in bile between the two sampling periods, a significant increase of 3 to 5-fold for HHCB was observed in June 2013 in fish from RR and BE, respectively. No significant temporal differences were observed for the other chemicals.

3.3. Organochlorine compounds in muscle

OC levels in the muscle of roach are shown in Figure 2 and summarised in Table 1. Detailed information on individual congeners analysed is provided in Table S2 (supplementary information). Regarding the spatial distribution, significant differences among sampling sites were observed for DDTs, HCHs, HCB and PCBs concentrations in October 2012; the highest concentrations were detected in fish from BE (1317 ± 105; 9 ± 2; 68 ± 1 and 1111 ± 22 ng/g d.w. of muscle, respectively). In June 2013, fish collected at downstream sites (BE, A) showed higher residues of PCBs and DDTs; whereas no significant differences among sampling sites were observed for the other compounds (HCHs, HCBs, PeCBs).

Regarding temporal trends, 4 to 9-fold higher concentrations of all OCs, with the exception of PeCB, were found in BE during barrier construction (2012) in comparison to the dredging period (2013) (Table 1). Similarly, DDTs, HCHs, HCB and PCBs were

found at higher concentrations prior than during dredging in fish collected in FL. No differences in OC concentrations in fish from RR, the reference site, were found between sampling periods.

DDTs were the most abundant pollutants in fish muscle, followed by PCBs, HCB, HCHs and PeCB. Among DDT metabolites, 4,4'-DDE was the dominant compound, comprising on average 50-70% of the total DDT content, while 4,4'-DDT contributed only to 4-7%. Samples from FL had higher 4,4'-DDT contribution than those from the other stations (Table S2). Among the seven PCB congeners determined, PCB153 (35%), PCB138 (22%) and PCB180 (15%), were the most prevalent. The dominant HCH isomer detected in muscle of all specimens was γ-HCH (52%), with the exception of fish collected in RR in October 2012, which showed higher proportion of β-HCH (80%).

3.4. Biochemical responses

In October 2012, EROD activity was significantly increased in fish from A (16 ± 6 pmol/min/mg protein), approximately 2-fold when compared to RR and FL, while no differences among sampling sites were detected for BFCOD and UGT activities (Fig. 3). In June 2013, the same tendency was observed, although only UGT activity was significantly elevated in fish from A (450 ± 32 pmolmin/mg protein) when compared to RR and FL (341 and 360 pmol/min/mg protein, respectively). On the other hand, no significant differences were observed for the selected biomarkers between 2012 and 2013, with the exception of EROD activity that was 1.7-fold higher in October 2012 than in June 2013 in fish from A. Principal Component Analysis (PCA) was used to classify sampling sites according to the observed biochemical responses, organochlorine

content in muscle and hydroxylated metabolites in bile. PCA rearranged the set of data in two factors, which together explained 78-79% of the total variance. During the first sampling EROD and UDPGT activities were not associated to OCs levels in muscle (HCB, HCH, PCB, DDT), which was probably due to the release of other contaminants (e.g. metals, alkylphenols (NP, OP)) in the area of FL and downstream (BE). In contrast, during dredging, an association of EROD and UGT activities with OCs levels in muscle (PCBs, DDTs) was observed (Fig. 2, Supplementary Information).

3.5. Histological analysis of the gonads

No significant abnormalities within gonad tissue of males or females were observed (Fig. 4). However, during barrier construction, female roach from the upper course of the river (RR and FL) had gonads at undeveloped stage (SMS-0), while females sampled downstream had gonads at advanced stages of sexual maturation. Thus, primary oocytes with perinuclear and cortical alveoli (SMS-I) and some secondary oocytes with yolk granules (SMS-II) were observed in females from BE, while 25% of females collected in station A had already oocytes completely filled with yolk granules (SMS-III). In contrast, no differences on maturation stage were observed for males, which had undeveloped gonads (SMS-0).

In June 2013, females from FL had undeveloped gonads (SMS-0), whereas those collected upstream (RR) and downstream (BE and A) had 50 to 100 % of the gonads classified as SMS-I. Regarding males, those collected upstream (RR, FL) had 50-70% of the gonads classified as SMS-0, while those collected downstream (BE, A) were mostly classified as SMS-I (early gametogenesis).

4. Discussion

Although sediments act as a sink for hydrophobic and persistent organic contaminants in aquatic systems, several processes such as physical disturbances induced by water currents, dredging or other activities can trigger the resuspension of contaminants back into the water column making them available to aquatic organisms (Latimer et al., 1999). Both, the construction of the wall and dredging activities could have enhanced the mobility of contaminants from FL towards the lower course of the Ebro River. The first sampling of roach took place in October 2012, two years from the beginning of the construction of the wall. When comparing OC levels in the muscle of roach collected downstream FL in October 2012 with those obtained in a previous study in 2006 (Table 1), a significant increase in the concentration of PCBs and DDTs was observed (up to 5.6-fold) in October 2012, and associated to the release of OCs during wall construction. The direct influence of FL contamination down to BE is further supported by the strong correlation observed between all analyzed OCs in fish from both stations, indicating a common pollution source (Table S3, Supplementary information). This strong correlation was not observed further downstream (A) and this was attributed to the presence of additional pollution sources together with the decoupling of the compounds during transport.

However, during the second sampling (June 2013), the construction of the barrier in FL had finished and sediments resuspended as a consequence of dredging were expected to be retained within the barrier. The effectiveness of the barrier was evidenced by the 4- to 9-fold lower concentrations of OCs found in muscle of fish from FL and BE in comparison to 2012. Although fish size/length was pretty homogenous, some differences were observed among sampling sites. Size differences could be a

source of variability regarding OC concentrations in muscle, and may have acted as a confounding factor. Some studies have reported a positive relationship between length and OC levels in freshwater fish, while others reported no clear relationship or even the opposite trend due to the so called dilution effect (Manchester-Neesvig et al., 2001; Covaci et al., 2006). In our study, no clear relationship between fish length and OC accumulation was observed.

PCBs 138, 153 and 180 were the most prevalent congeners in roach muscle (Table S2). These congeners have a high degree of chlorination (hexa and hepta-PCB) and consequently greater tendency to adsorb in sediments and to bioaccumulate in organisms than the less chlorinated ones. Regarding DDTs, 4,4'-DDE was the dominant isomer in all samples; its predominance is indicative of old DDT residues that progressively degrade into 4,4'-DDE (Shaw et al., 2005). Another characteristic feature is the significant amount of DDDs found in FL and its area of influence, which has been also reported by other authors (Huertas, 2015). These relatively higher concentrations of DDDs are due to the anaerobic conditions of the sediments in FL, which facilitate the transformation of DDT into DDD instead of DDE. The highest percentage of DDDs was observed in samples from FL and downstream sites in 2012, with a strong decrease in 2013 in BE and A (Fig. 5). Values in the reference site (RR) were lower and remain uniform among samplings. These results further evidence a strong pollution load from FL to downstream areas in 2012 (during wall construction) that was significantly reduced in 2013 (dredging).

While significant spatial and temporal differences were detected for OCs in roach muscle, bile analysis did not evidence substantial differences among sites or samplings, mainly due to a high inter-individual variability. High concentrations of octyl- and nonylphenol were previously detected in the bile of carps from FL (NP: 16.5

396 and OP: 0.3 µg/g of bile), indicating a continuous and significant input of these
397 compounds in the area (Lavado et al., 2006). APs act as endocrine disruptors on aquatic
398 organisms by binding to the estrogen receptor. Consequently, presence of immature
399 cells into the lumen, macrofage aggregates in testes, and depressed levels of testosterone
400 and estradiol in plasma were observed in male carps, while delayed maturation was
401 reported in females (Lavado et al., 2006). Moreover, high concentrations of OCs,
402 namely PCBs (95 ng/g w.w), DDTs (29 ng/g w.w) and HCB (1.65 ng/g w.w) were
403 detected in the muscle of these carps, suggesting that not only APs, but also OCs might
404 be responsible for the significant endocrine alterations detected (Lavado et al., 2004;
405 2006). In the present study, no significant alteration in roach gonads was observed.
406 Certainly, biliary levels of NP and OP were 3 to 5-fold lower in roach than in carp, and
407 OCs residues in muscle, including PCBs, HCBs and DDTs were up to 43-, 2-, and 8-
408 fold lower in roach, indicating reduced exposure in comparison to the carp's study.
409 Nonetheless, NP levels determined in the bile of roach (450-2250 ng/mL) were
410 relatively high in comparison to those reported in deep-sea fish (17-107 ng/g), but 3- to
411 5-fold lower than the concentrations reported in the bile of roach exposed to effluents
412 from waste water treatment plants (Fenlon et al., 2010; Koenig et al., 2013). The
413 histological examination of the gonads indicated that a delay in gonad maturation might
414 have occurred in female roach from downstream areas (BE, A) during wall construction.
415 Females from these areas had still gonads at advanced stages of sexual maturation,
416 while males and females from FL an RR, less exposed to organochlorines and other
417 pollutants released during wall construction, had undeveloped gonads. In contrast, no
418 evidence of maturation delay was observed during the second sampling, as all the
419 individuals examined had gonads at SMS-0 or SMS-1 (undeveloped or early

420 gametogenesis). However, due to the reduced number of males and females examined,
421 these results should be interpreted with caution.

422 Among biomarkers, EROD activity has been successfully used as biomarker of
423 exposure to a wide variety of organic pollutants, including polycyclic aromatic
424 hydrocarbons, dioxin-like PCBs and many others (Whyte et al., 2000). EROD activity
425 was significantly elevated in roach from downstream sites (BE, A) during wall
426 construction, while during dredging, EROD activity in the liver of fish from
427 downstream sites had decreased, and no significant differences among sites was
428 observed. Similarly, the determination of BFCOD activity did not show significant
429 differences among sampling sites. BFCOD is a measure of CYP3A activity, which is
430 induced by steroids, bile acids and different xenobiotics, e.g. pharmaceuticals,
431 pesticides, among others. Reports on the induction of BFCOD activity in fish are scarce
432 and few studies have applied this biomarker in field studies (Quesada-García et al.,
433 2013; Habila et al., 2017). Regarding UGT activity, a significant increase was observed
434 in roach from station A during dredging. Generally, the transcriptional induction of
435 UGTs via AhR has been described in the liver of fish (Christen and Fent, 2014). This
436 leads to induction of many UGT isoforms together with CYP1A enzymes that will
437 conjugate a broad range of both endogenous (bilirubin, bile acids, estrogens, androgens,
438 thyroid hormones, etc.) and exogenous (phenols, non-steroidal anti-inflammatory drugs,
439 etc.) substrates. We cannot discard that the unresponsive profile of the enzymatic
440 activities assessed be a sign of the presence of metals (Cu, Hg, Cd), which might
441 regulate xenobiotic-induced AhR transcription (Chen and Chan, 2016), as high
442 concentrations of Cd and Hg have been reported in biota from FL area and downstream
443 areas (Lavado et al., 2006; Alcaraz et al., 2011).

Overall, the construction works of the barrier in Flix produced the resuspension and subsequent mobilization of contaminants (OCs) downstream as evidenced by the high accumulation of organochlorinated compounds found in the muscle of fish in BE in October 2012, during wall construction. The OC profiles were strongly related to those detected in FL, indicating a common pollution source for OCs. This observation was supported by increased EROD activity in fish from downstream areas. However, during dredging (June 2013), the wall efficiently retained the resuspended pollutants as shown by the decreased concentration of OCs in muscle of fish in FL and downstream sites in comparison to previous studies. In addition, the lack of induction of EROD activity in fish from downstream sites during dredging indicates no significant release of CYP1A inducers and further proves the effectiveness of the barrier.

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596

597 Table 1. Total concentrations of OCs (DDTs, HCHs, PCBs, HCB, PeCB) reported in
598 muscle of *Rutilus rutilus* (ng/g of dry weight) collected in Flix and downstream areas.
599

	Flix			50-65 km downstream			90-100 km downstream		
	2006[a]	2012	2013	2006[a]	2012	2013	2006[a]	2012	2013
∑DDT	477	111±46	19±3	227	1317±105	242±53	226	364±45	212±27
∑HCH	5.0	3±0.3	0.5±0.4	3.5	9±2	1.3±0.7	2	2±1	1.2±0.6
∑PCB	408	114±57	11±4	196	1111±22	305±67	141	183±53	237±18
HCB	1292	20±12	4±2	37	68±1	7.2±3	39	9±1	7±1
PeCB	116	7±3	1±0.2	2.5	2.4±0.02	1.3±0.8	2.5	0.3±0.03	0.6±0.4

600
601 [a] Data from Huertas (2015) originally reported as ng/g wet weight and multiplied by a factor of 5 to
602 estimate concentrations in dry weight (considering water content of 80% in muscle tissue).

Figure 1. Biliary levels of alkylphenols (4-nonylphenol (NP) and 4-*tert*-octylphenol (OP)), hydroxylated PAHs (1-naphthol, 1-pyrenol) and galaxolide (HHCB) in *Rutilus rutilus* collected in October 2012 and June 2013 along the Ebro River. Values are expressed as mean ± SEM (n = 5-9). Distinct letters indicate significant differences between sites and * indicates significant differences between October 2012 and June 2013 samplings ($p < 0.05$).

Figure 2. Levels of PCBs, DDTs, HCHs and HCB (ng/g dry weight) analysed in muscle of *Rutilus rutilus* collected in October 2012 and June 2013 in RR, FL, BE and A. Values are expressed as mean ± SEM (n = 2-3). Each sample corresponds to a pool of 3 individual fish. Distinct letters indicate significant differences between sites and * indicates significant differences between October 2012 and June 2013 samplings ($p < 0.05$).

Figure 3. (A) EROD, (B) BFCOD and (C) UGT activities determined in the liver of *Rutilus rutilus* collected in October 2012 and June 2013 along the Ebro river (RR, FL, BE and A). Values are expressed as mean ± SEM (n = 6-12). Distinct letters indicate significant differences between sites ($p < 0.05$).

Figure 4. Percentage of individuals detected at different stages of gonad maturation. A: females; B: males.

Figure 5. Percentage of 4,4'-DDD out of total DDT content detected in muscle tissue of *Rutilus rutilus* collected in October 2012 and June 2013 along the Ebro river (RR, FL, BE and A). Horizontal line shows median values and box is interquartil range.

Figure 1.

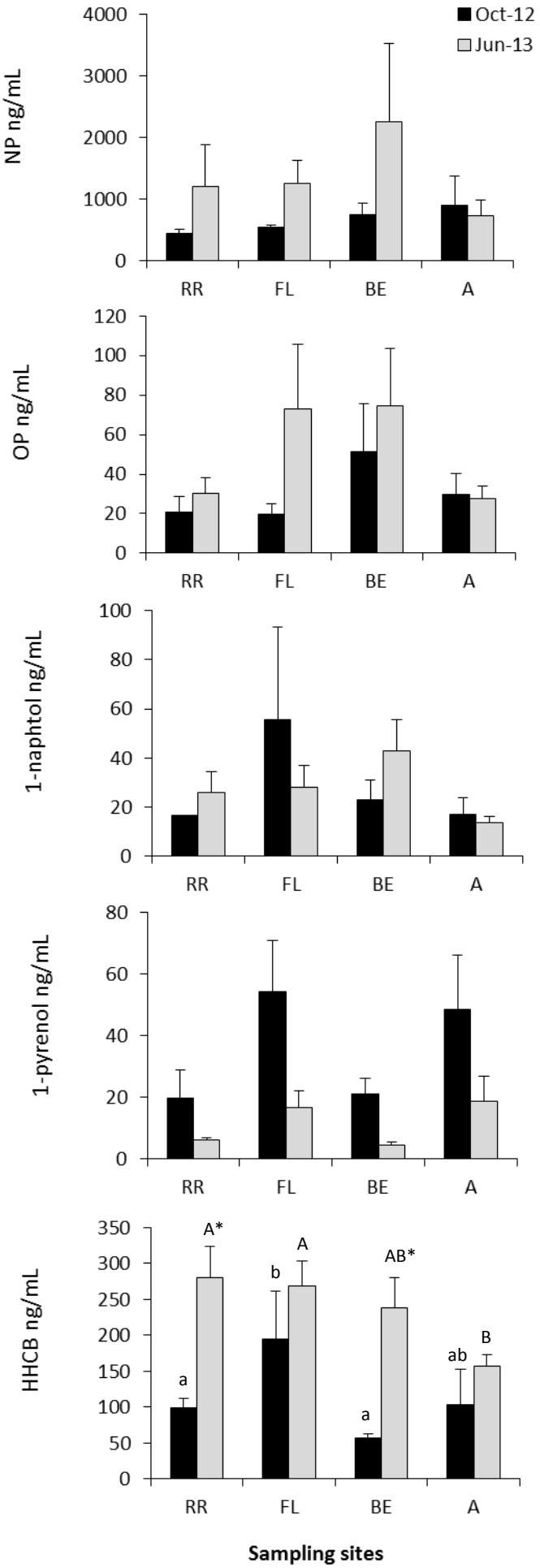

Oct-12
Jun-13
NP ng/mL
4000
3000
2000
1000
0
RR
FL
BE
A
OP ng/mL
120
100
80
60
40
20
0
1-naphtol ng/mL
1-pyrenol ng/mL
HHCB ng/mL
350
300
250
200
150
100
50
A*
A
b
AB*
a
ab
B
Sampling sites

Figure 2.

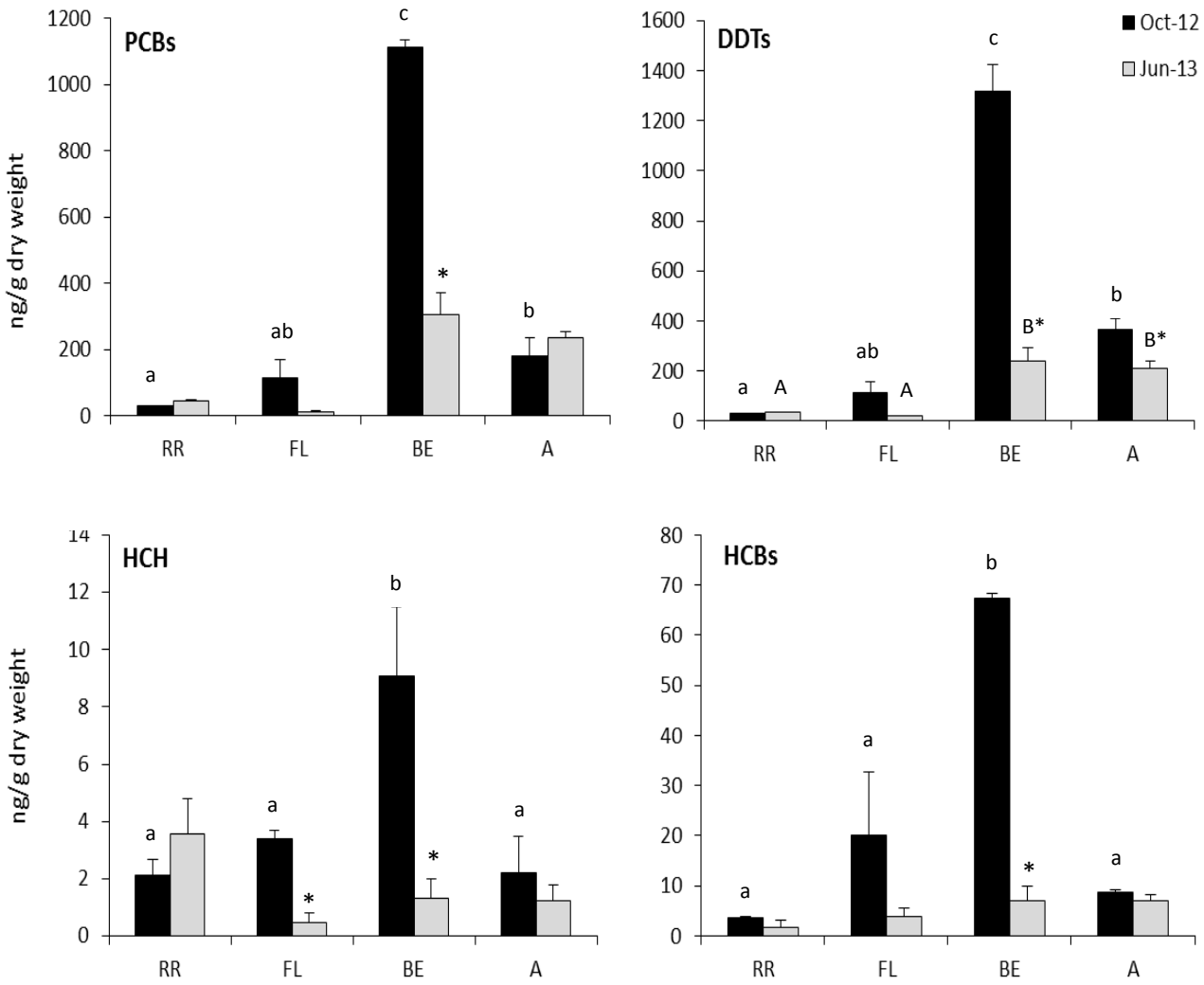


Figure 3.

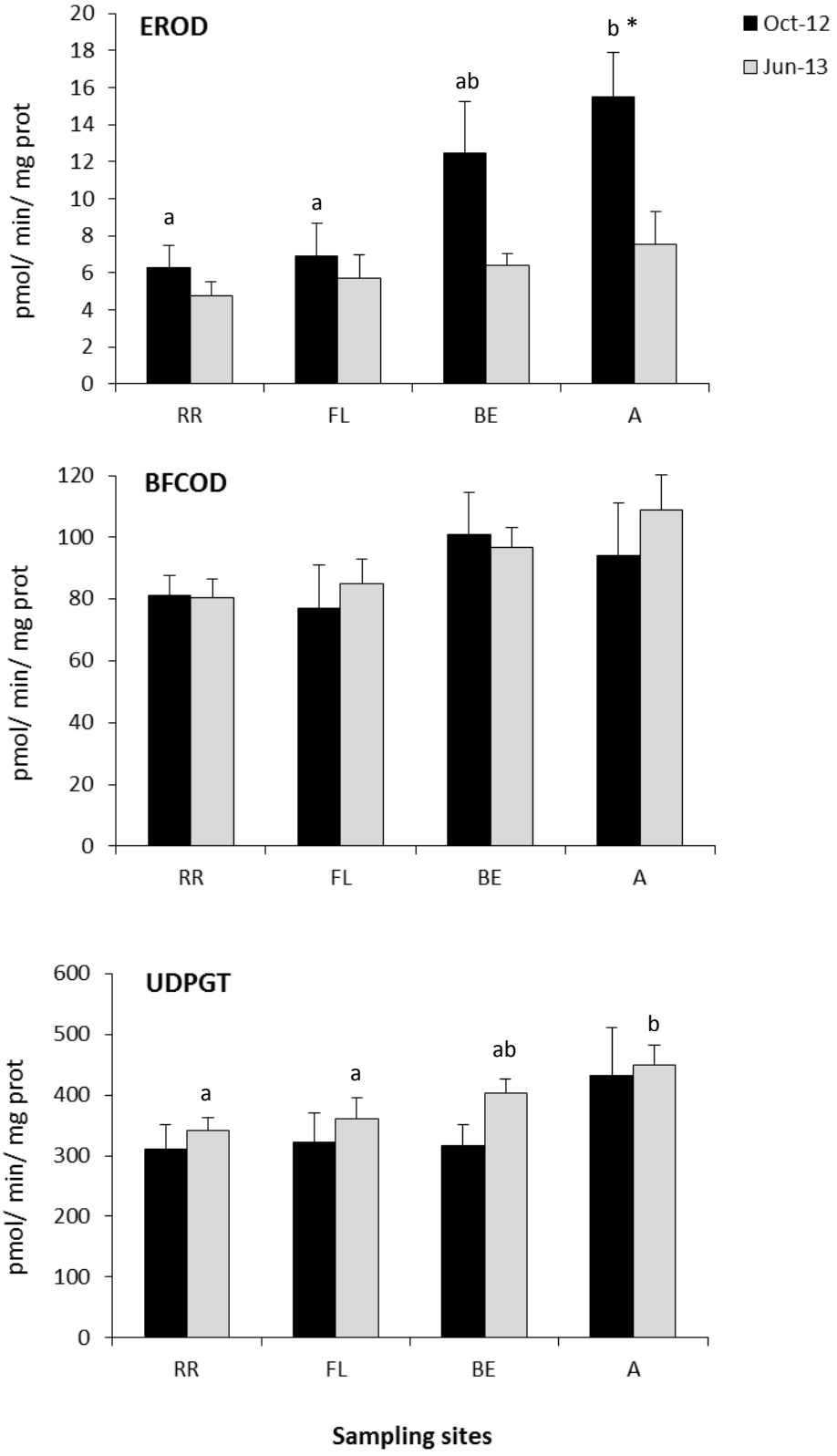
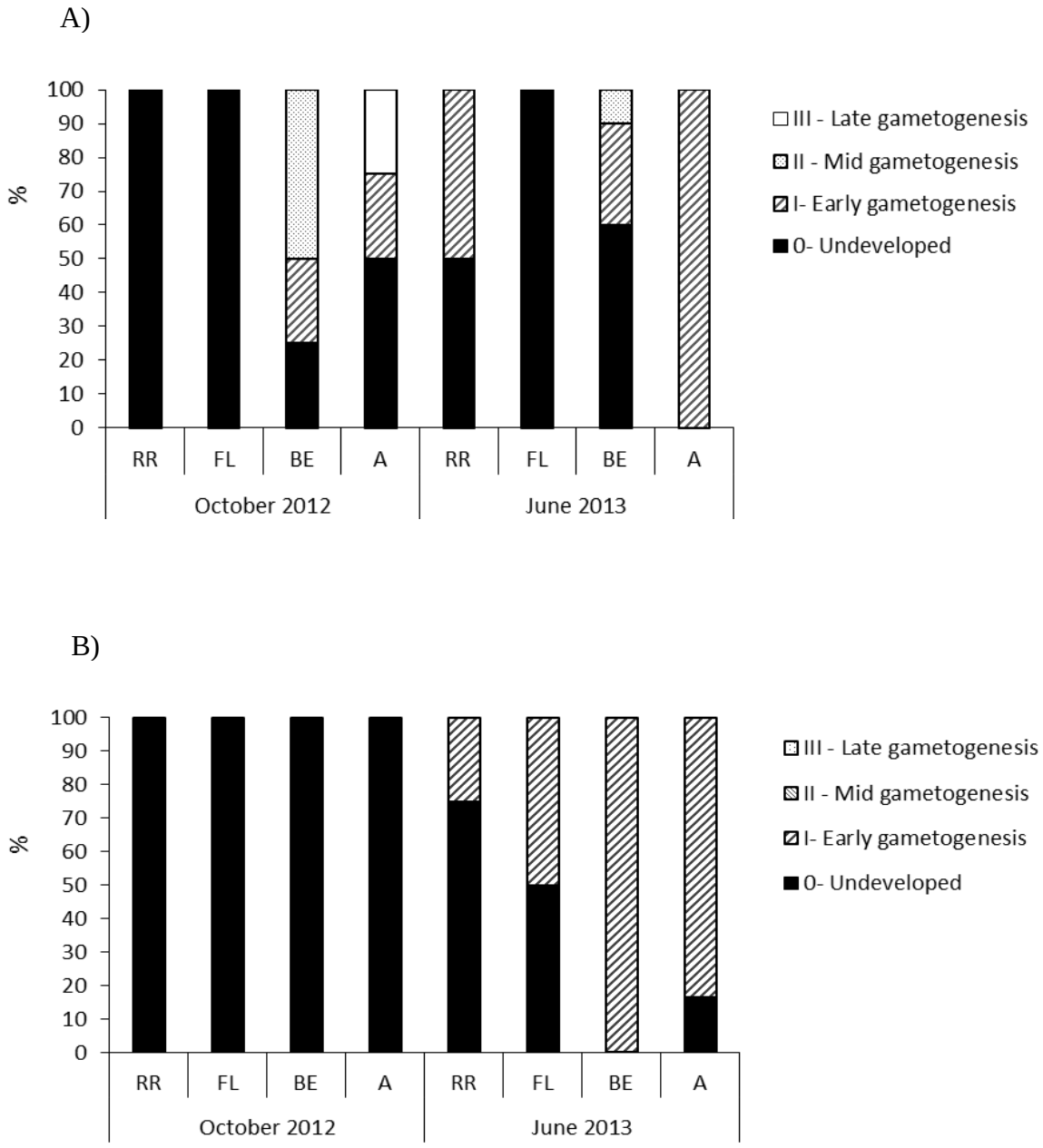

EROD
Oct-12
Jun-13
pmol/ min/ mg prot
a
a
ab
b *
RR
FL
BE
A
BFCOD
UDPGT
Sampling sites

Figure 4.


A)
100
90
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70
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50
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30
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10
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%
RR
FL
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A
RR
FL
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A
October 2012
June 2013
III - Late gametogenesis
II - Mid gametogenesis
I- Early gametogenesis
0- Undeveloped
B)
100
90
80
70
60
50
40
30
20
10
0
%
RR
FL
BE
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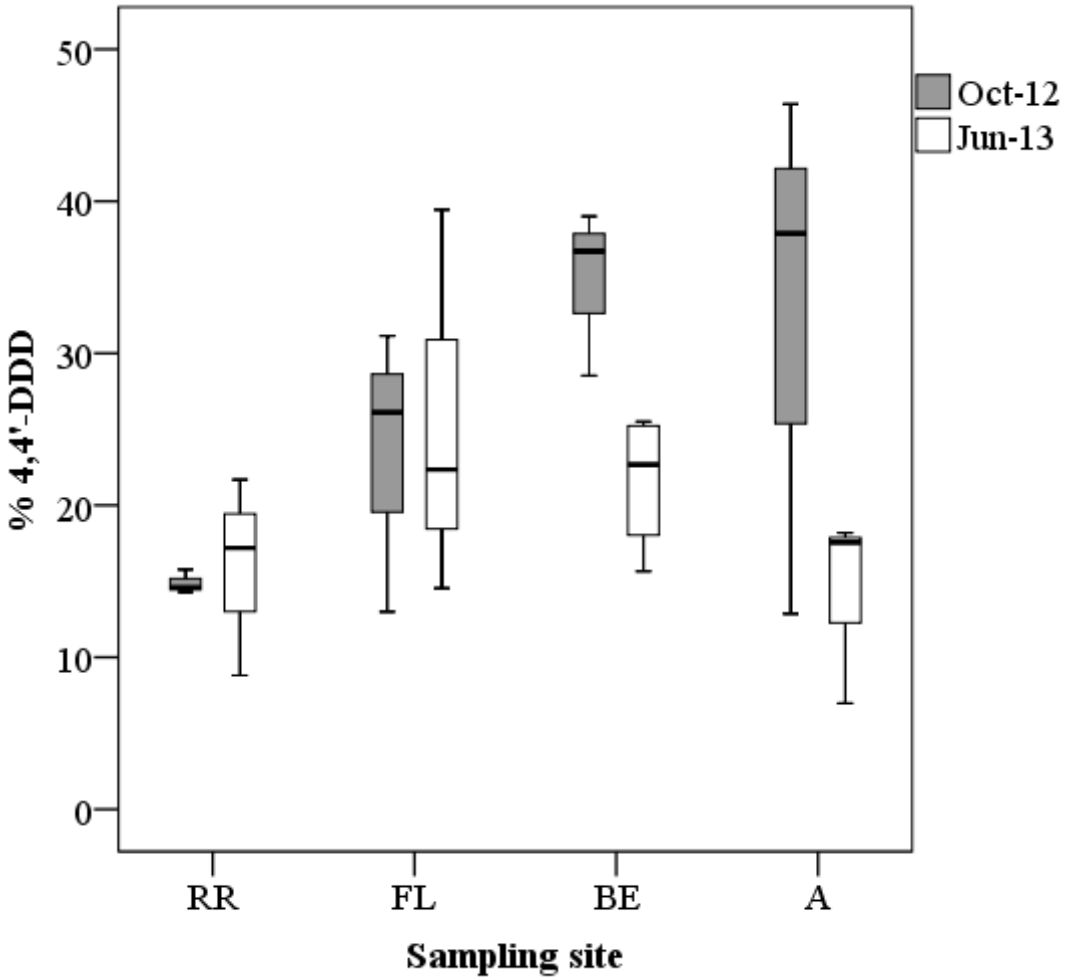
RR
FL
BE
A
October 2012
June 2013
III - Late gametogenesis
II - Mid gametogenesis
I- Early gametogenesis
0- Undeveloped

Figure 5.



Supplementary information

The combined use of chemical and biochemical markers to assess the effect of dredging in the lower course of the Ebro River

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Table S1. Total length, body weight and condition factor (CF) of *Rutilus rutilus* collected in the Ebro River in October 2012 and June 2013. Values are mean ± SEM (n = 6-12). Distinct letters indicate significant differences between sites ($p < 0.05$).

	Sampling	Station			
		RR	FL	BE	A
Total length (cm)	Oct-12	13.8 ± 0.3^{a}	13.1 ± 0.3^{ab}	12.2 ± 0.2^{ab}	11.5 ± 0.6^{b}
	Jun-13	12.3 ± 0.3^{a}	11.6 ± 0.1^{a}	13.6 ± 0.1^{b}	16.3 ± 0.3^{c}
Body weight (g)	Oct-12	29.5 ± 0.7^{a}	22.2 ± 1.7^{b}	22.7 ± 1.0^{b}	22.5 ± 4.0^{b}
	Jun-13	30.0 ± 2.6^{ab}	21.3 ± 1.3^{a}	37.1 ± 1.3^{b}	67.5 ± 3.5^{c}
CF	Oct-12	1.2 ± 0.1^{a}	0.97 ± 0.1^{a}	1.2 ± 0.04^{a}	1.1 ± 0.1^{a}
	Jun-13	1.5 ± 0.05^{a}	1.3 ± 0.1^{a}	1.5 ± 0.03^{a}	1.5 ± 0.04^{a}

CF calculated as (weight / length^3) x 100.

Table S2**.** Concentration of different OCs detected in muscle of *Rutilus rutilus* collected in October 2012 and June 2013 along the Ebro River. Values are expressed in ng/g d.w. as mean ± SEM (n = 3). Each sample corresponds to a pool of 3 individual fish.

	2012				2013			
	RR	FL	BE	A	RR	FL	BE	A
HCB	3.5 ± 0.2	20.2 ± 7.0	47.3 ± 11.3	8.6 ± 0.4	1.7 ± 0.8	3.8 ± 0.96	7.1 ± 1.6	7.1 ± 0.6
α-HCH	n.d	n.d	1.1 ± 0.1	0.5 ± 0.05	n.d	n.d	n.d	n.d
β-HCH	1.8 ± 0.2	0.9 ± 0.08	1.6 ± 0.4	0.6 ± 0.07	0.7 ± 0.1	0.3 ± 0.1	0.4 ± 0.08	0.3 ± 0.05
γ-HCH	0.2 ± 0.08	1.4 ± 0.03	3.8 ± 1.5	1.1 ± 0.4	2.4 ± 0.7	0.1 ± 0.06	0.8 ± 0.3	0.7 ± 0.2
δ-HCH	0.2 ± 0.01	0.6 ± 0.06	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.005	0.2 ± 0.06	0.3 ± 0.01	0.3 ± 0.07
2,4'-DDE	1.05 ± 0.3	2.9 ± 0.4	5.9 ± 1.8	8.5 ± 2.3	3.3 ± 0.8	0.7 ± 0.3	14.3 ± 7.5	4.5 ± 2.2
4,4´-DDE	20.2 ± 0.9	63.2 ± 12.3	497.0 ± 102.4	197.3 ± 26.4	19.6 ± 1.8	8.3 ± 0.3	136.5 ± 21.1	151.1 ± 12.1
2,4'-DDD	4 ± 0.3	14.3 ± 4.05	137.3 ± 33.9	34.1 ± 5.7	4.3 ± 0.4	4.8 ± 0.9	37.5 ± 8.1	18.2 ± 2.8
4,4'-DDD	3.5 ± 0.1	25.2 ± 8.06	282.9 ± 62.6	93.5 ± 14.7	4 ± 0.4	3.4 ± 0.7	42.7 ± 8.4	29.1 ± 5.6
2,4'-DDT	n.d	n.d	n.d	13.3 ± 1.6	n.d	n.d	3.4 ± 0.8	n.d
4,4'-DDT	n.d	5.8 ± 0.9	42.8 ± 5.3	21.9 ± 2.3	2.5 ± 0.3	1.2 ± 0.06	10.1 ± 2.5	9.1 ± 0.5
PeCB	2.6 ± 0.7	6.7 ± 1.6	3.8 ± 0.8	7.04 ± 3.8	2.1 ± 0.9	0.7 ± 0.09	1.3 ± 0.5	0.6 ± 0.2
PCB 101	5.7 ± 0.5	11.5 ± 2.6	68.5 ± 14.2	19.9 ± 0.4	3.6 ± 0.5	2.4 ± 0.8	25.7 ± 3.04	16 ± 2.8
PCB 28	1.5 ± 0.2	5.4 ± 0.4	58.7 ± 13.4	10.3 ± 1.3	2.2 ± 0.2	0.8 ± 0.1	12.9 ± 2.2	8.6 ± 0.6
PCB 52	3.9 ± 0.1	5.4 ± 0.7	52.1 ± 11.6	12.6 ± 0.5	3.3 ± 0.3	0.6 ± 0.1	15.5 ± 2.5	14.9 ± 1.05
PCB 118	2.5 ± 0.1	8.7 ± 1.5	44.5 ± 9	11.7 ± 1.9	6.2 ± 1	0.8 ± 0.4	32.7 ± 10.5	21.6 ± 3.9
PCB 153	7.2 ± 0.3	37.2 ± 12	293.4 ± 59.8	68.1 ± 16.1	14.7 ± 1	1.4 ± 0.03	97.6 ± 11	88.2 ± 4.2
PCB 138	5.4 ± 0.3	24.4 ± 6.9	179.7 ± 36.9	38.4 ± 9.3	10.3 ± 0.9	2.2 ± 0.6	72.5 ± 10.3	53 ± 2.4
PCB 180	2.9 ± 0.9	21.1 ± 8.6	117.4 ± 24.4	28.3 ± 5.8	5.5 ± 0.3	2.4 ± 0.7	48.7 ± 5.8	34.9 ± 3.01
∑DDTs	28.8 ± 1.6	111.3 ± 25.9	965.8 ± 202.5	364.2 ± 25.4	33.6 ± 2.3	18.9 ± 1.6	242 ± 29.7	212 ± 15.3
∑HCHs	2.1 ± 0.3	3.4 ± 0.2	6.9 ± 1.8	2.2 ± 0.7	3.6 ± 0.7	0.5 ± 0.2	1.3 ± 0.4	1.2 ± 0.3
∑PCBs	29.2 ± 0.8	113.8 ± 32.4	814.3 ± 167.8	182.7 ± 30	45.8 ± 2.5	10.7 ± 2.5	305.3 ± 37.7	237.2 ± 10.1

n.d: below detection limit.

Table S3. Pearson correlation coefficients between log-transformed concentrations of OCs in the sampling sites. PeCB is not included as it showed no relationship with the other contaminants in any site.

			HCB	DDTs	HCHs	PCBs		HCB	DDTs	HCHs	PCBs
RIBARROJA	HCB	*r*		-0.558	-0.648	-0.582	**BENIFALLET**		0.991**	0.900**	0.964**
		p		0.25	0.164	0.225			0.000	0.006	0.000
		N		6	6	6			7	7	7
	DDTs	*r*			0.846*	0.728				0.890**	0.968**
		p			0.034	0.101				0.007	0.000
		N			6	6				7	7
	HCHs	*r*				0.662					0.942**
		p				0.152					0.001
		N				6					7
	PCBs	*r*									
		p									
		N									
FLIX	HCB	*r*		0.923**	0.797	0.948**	**AMPOSTA**		0.271	0.689	-0.086
		p		0.009	0.057	0.004			0.604	0.13	0.871
		N		6	6	6			6	6	6
	DDTs	*r*			0.802	0.985**				0.425	0.017
		p			0.055	0.000				0.401	0.974
		N			6	6				6	6
	HCHs	*r*				0.884*					0.269
		p				0.019					0.606
		N				6					6
	PCBs	*r*									
		p									
		N									

r, Pearson correlation coefficient; *p*, significance level; N, number of samples. *Statistically significant at 95% confidence level.

**Statistically significant at 99% confidence level.

Figure S1. Map of the sampling sites. RR: Ribarroja; FL: Flix; BE: Benifallet; A: Amposta.

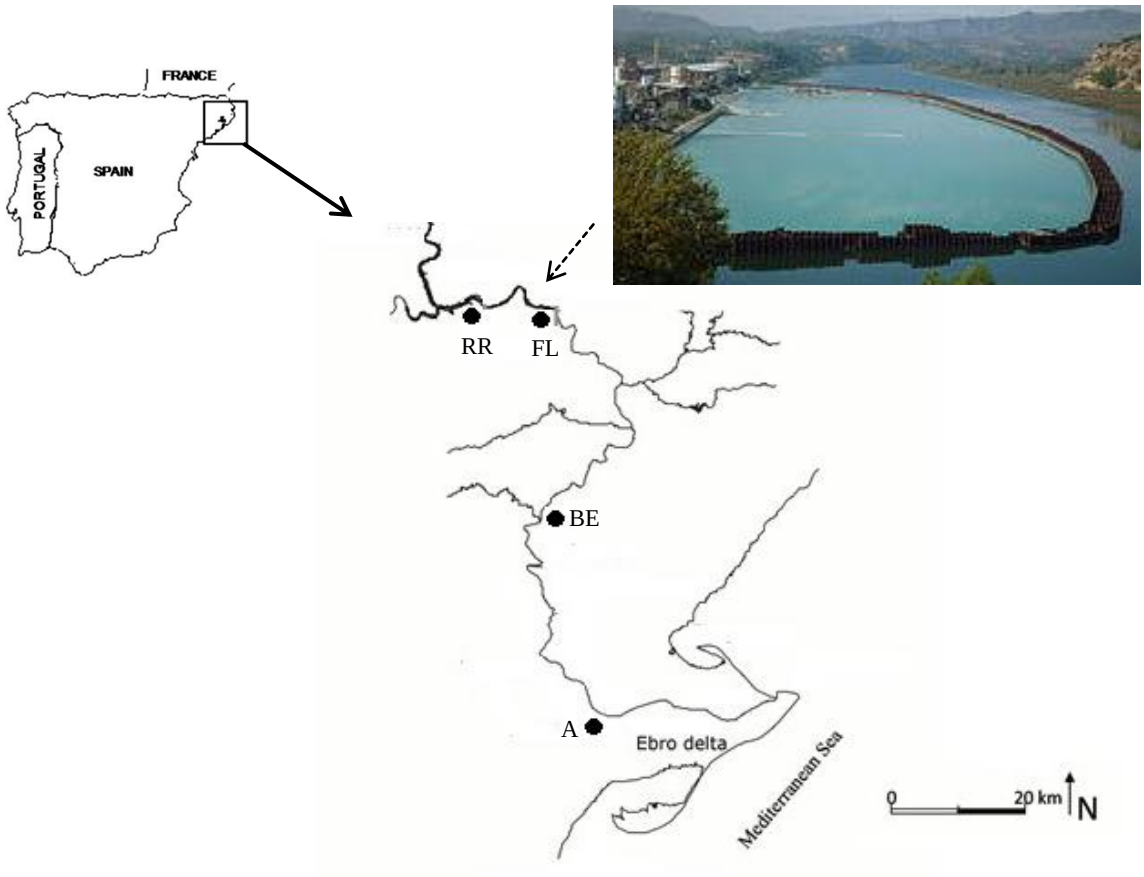

Figure 2. PCA analysis of the data shows that during the first sampling (A) EROD and UDPGT activities were not associated to OCs levels in muscle (HCB, HCH, PCB, DDT), which is probably due to the release of other contaminants (e.g. metals, alkylphenols (NP, OP)) in the area of FL and downstream (BE). The exposure of fish to those compounds (together with OCs) will have an inhibitory effect on CYP1A activity. In contrast, during dredging (B) the association of EROD and UGT activities with OCs levels in muscle (PCBs, DDTs) was observed. PY: 1-pyrenol; NAPH: 1-naphthol.